Fractional Laser-Assisted Delivery of Methyl Aminolevulinate: Impact of Laser Channel Depth and Incubation Time

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Background and Objectives: Pretreatment of skin with ablative fractional lasers (AFXL) enhances the uptake of topical photosensitizers used in photodynamic therapy (PDT). Distribution of photosensitizer into skin layers may depend on depth of laser channels and incubation time. This study evaluates whether depth of intradermal laser channels and incubation time may affect AFXL-assisted delivery of methyl aminolevulinate (MAL).

Materials and Methods: Yorkshire swine were treated with CO_2 AFXL at energy levels of 37, 190, and 380 mJ/laser channel and subsequent application of MAL cream (Metvix®) for 30, 60, 120, and 180 minutes incubation time. Fluorescence photography and fluorescence microscopy quantified MAL-induced porphyrin fluorescence (PpIX) at the skin surface and at five specific skin depths (120, 500, 1,000, 1,500, and 1,800 μm).

Results: Laser channels penetrated into superficial (~300 μm), mid (~1,400 μm), and deep dermis/upper subcutaneous fat layer (~2,100 μm). Similar fluorescence intensities were induced at the skin surface and throughout skin layers independent of laser channel depth (180 minutes; $P < 0.19$). AFXL accelerated PpIX fluorescence from skin surface to deep dermis. After laser exposure and 60 minutes MAL incubation, surface fluorescence was significantly higher compared to intact, not laser-exposed skin at 180 minutes (AFXL-MAL 60 minutes vs. MAL 180 minutes, 69.16 a.u. vs. 23.49 a.u.; $P < 0.01$). Through all skin layers (120–1,800 μm), laser exposure and 120 minutes MAL incubation induced significantly higher fluorescence intensities in HF and dermis than non-laser exposed sites at 180 minutes (1,800 μm, AFXL-MAL 120 minutes vs. MAL 180 minutes, HF 14.76 a.u. vs. 6.69 a.u. and dermis 6.98 a.u. vs. 5.87 a.u.; $P < 0.01$).

Conclusions: AFXL pretreatment accelerates PpIX accumulation, but intradermal depth of laser channels does not affect porphyrin accumulation. Further studies are required to examine these findings in clinical trials. Lasers Surg. Med. © 2012 Wiley Periodicals, Inc.

Key words: drug delivery; fractional CO_2 laser; photosensitizer

INTRODUCTION

Non-melanoma skin cancer (NMSC) is the most common cancer in humans [1,2]. Photodynamic therapy (PDT) is an attractive non-invasive treatment of actinic premalignant lesions and selected NMSCs because large skin areas and multiple lesions can be treated with superior cosmetic outcomes [3–7]. In PDT a topical photosensitizer is applied to the skin. The photosensitizer is converted into the light active fluorescent protoporphyrin IX (PpIX) through the heme biosynthesis. Accumulation of PpIX is intensified in abnormal cells and upon photoactivation, PDT therefore causes targeted destruction of dysplastic and neoplastic tissue [4,8].

In recent years, *in vitro* and *in vivo* experimental studies have demonstrated that pretreatment of the skin with ablative fractional lasers (AFXL) enhances the uptake of topically applied photosensitizers and facilitates intracutaneous distribution into deeper skin layers [9–12]. AFXL disrupts the skin barrier by creating microscopic vertical channels of ablated tissue surrounded by a zone of coagulated tissue. Each channel and its surrounding coagulation zone constitute a microthermal zone (MTZ). Ablative fractional procedures are available with carbon dioxide (CO_2; 10,600 nm), erbium:yttrium aluminum garnet (Er:YAG; 2,940 nm), and yttrium scandium gallium garnet (YSGG; 2,790 nm) lasers [13–15]. All of these infrared wavelengths are absorbed by water. Different absorption

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characteristics of wavelengths as well as varying laser settings such as power, pulse duration, and laser density cause different laser–tissue interactions, meaning that dimensions of generated laser channels and surrounding coagulation zone can be varied [16,17].

Treatment response after conventional PDT depends on type and thickness of lesions with reduced cure rates for thicker elements [4,6,18–24]. The limited treatment response is considered due to an insufficient PDT response in the deeper skin layers [25]. A recent *in vivo* animal study illustrated that pretreatment with AFXL facilitated an intensified PDT response in deeper skin layers [10] and recent clinical studies on AFXL-assisted PDT for actinic keratoses and NMSC have demonstrated intensified treatment responses [26,27]. AFXL-assisted PDT may enable intensified treatment of thicker lesions due to a deeper distribution of photosensitizer. Furthermore, the accelerated uptake of photosensitizer due to disrupted skin barrier may reduce the incubation time required for obtaining sufficient PpIX accumulation.

The knowledge on optimal laser settings and interaction with photosensitizer incubation time for AFXL-assisted PDT is limited. Thus, the importance of laser channel depth, density, and coagulation zone remains unclear [11,12]. Two experimental animal studies conducted on porcine and murine skin, have so far evaluated the impact of varying MTZ penetration depth and results have not been consistent [11,12]. Both studies were conducted with fractional Er:YAG laser systems that drilled superficial laser channels into stratum corneum and down to mid epidermis (range of laser channels approximately 10–70 μm).

Today, no information exists on the importance of intradermal laser channel depth and fractional CO_2 laser settings for distribution of photosensitizer throughout epidermal and dermal skin layers. Moreover, information on optimal incubation times for AFXL-facilitated delivery of photosensitizers is required.

This *in vivo* study was conducted with a fractional CO_2 laser. Objectives were to investigate the importance of varying intradermal laser channel depths for AFXL-assisted delivery of a topical photosensitizer, methyl aminolevulinate (MAL), and to examine PpIX accumulation after different incubation times. The clinical perspective is to optimize parameters for AFXL-assisted PDT.

MATERIALS AND METHODS

Animals

The study was approved by the Massachusetts General Hospital Institute Subcommittee for Research Animal Care. Two female Yorkshire swine (16 weeks old, 54 kg) were anesthetized using telazol/xylazine (4.4 and 2.2 mg/kg i.m.) and isoflurane (2% with oxygen 3.0 L/minute) after overnight fasting and temperature controlled blankets were used to keep their core temperature stable. At the end of experiments euthanasia was performed with phenobarbital 100 mg/kg i.v.

Study Design

The study evaluated accumulation of MAL-induced PpIX fluorescence after AFXL pretreatment with three different intradermal laser channel depths. Moreover, the importance of incubation time was examined for deep dermal laser channels. One flank of each pig was shaved, rinsed with water, dried with towels, and rinsed twice with isopropanol before demarcation of tests areas. Test areas were exposed to fractional CO_2 laser at three different energies. Topical MAL (Metvix®, Galderma, La Défense, Cedex, France) or placebo cream (Unguentum M, Almirall Hermal GmbH, Reinbek, Germany) was applied according to the different interventions groups (Table 1). All test areas were occluded and experiments were conducted under dim light conditions. Digital florescence photographs were taken to quantity porphyrin florescence at the skin surface and fluorescence microscopy of frozen horizontal sectioned biopsies quantified the porphyrin fluorescence in dermis and hair follicles (HF) at five different skin depths (120, 500, 1,000, 1,500, and 1,800 μm).

Laser Treatment

AFXL treatment was performed with a continuous wave prototype CO_2 laser built by Reliant (now Solta, Palo Alto, CA) with a scanning device (48 series laser, SH Series Marketing Head, Model SH 3X-U/479, Synradinc., Mukilteo, WA). Single holes were created by pulse durations of 3 milliseconds and at laser powers of 12.3 and 31.5 W (i) 12.3 W, single pulse (ii) 31.5 W, two stacked pulses, and (iii) 31.5 W, four stacked pulses, delivering 37, 190, and 380 mJ/MTZ respectively. An external powermeter (Nova II Ophir, sensorP/N 1Z02604, serial no. 503856, Tel Aviv, Israel) was used to monitor the delivered energy at the three settings.

Surface Fluorescence

Digital surface fluorescence photography was performed immediately after laser exposure and at 30, 60, 120, and 180 minutes after cream application. Each test area was photographed only once to minimize light exposure. Photographs were taken with a 400 nm excitation filter and 630 nm emission interference filter, mounted on a digital camera (Nikon D70, 60 mm, F 2.8 lens) with a fixed focal distance of 30 cm. Four fluorescent standards (Red-Tag, Cypress, CA) were incorporated in the corners of each photo to standardize for frame-to-frame variations. In each picture, fixed regions of interest were selected around the individual laser holes (250 × 250 pixels corresponding to 9 mm × 9 mm) and the mean fluorescence intensity was calculated using MabLab (MatLab 7.0.1, MathWorks, Natick, MA).

Dermal and Hair Follicle Fluorescence

A total of 160 biopsies (10 from each of 16 interventions, Table 1) were taken from control and test areas at different time points from 30 to 180 minutes after cream application. Biopsies were embedded in OCT medium (Sakura

TABLE 1. Median Fluorescence Intensities in Hair Follicles and Dermis

		Skin depth				
Intervention	Time (minutes)	120 μm (HF/D)	500 μm (HF/D)	1,000 μm (HF/D)	1,500 μm (HF/D)	1,800 μm (HF/D)
1. MAL + AFXL (37 mJ)	180	22.98/8.94	41.39/7.96	40.03/8.54	35.04/8.36	24.03/7.84
2. MAL + AFXL (190 mJ)	180	32.29/9.36	34.38/8.43	32.36/8.84	33.15/8.16	28.90/8.19
3. MAL + AFXL (380 mJ)	30	7.47/6.38	6.38/6.31	6.55/6.15	6.77/6.03	6.51/5.86
4. MAL + AFXL (380 mJ)	60	12.90/6.08	9.96/5.96	8.68/6.06	7.55/5.73	7.20/5.92
5. MAL + AFXL (380 mJ)	120	12.82/7.69	20.61/7.24	18.46/7.35	19.10/6.90	14.76/6.98
6. MAL + AFXL (380 mJ)	180	31.55/9.83	36.97/9.23	29.87/9.09	33.31/8.89	32.00/8.75
7. MAL	30	6.41/5.67	6.09/5.63	5.94/5.44	6.10/5.84	6.45/5.97
8. MAL	60	6.69/6.16	6.14/6.03	6.11/6.13	6.21/6.08	5.83/5.91
9. MAL	120	6.87/5.87	6.30/5.92	6.20/5.88	5.78/5.63	6.38/5.71
10. MAL	180	6.47/6.38	6.16/6.01	6.68/5.99	6.93/5.86	6.69/5.87
11. AFXL (37 mJ)	180	6.81/6.19	6.25/5.84	6.24/5.64	5.93/5.85	6.03/5.69
12. AFXL (190 mJ)	180	6.47/6.02	6.11/5.83	6.00/5.73	5.86/5.61	5.92/5.77
13. AFXL 380 mJ)	180	6.38/5.91	5.88/5.88	6.32/5.96	6.00/5.70	5.94/5.76
14. Ung. M + AFXL (380 mJ)	180	5.02/6.11	4.99/5.79	4.89/5.52	4.61/5.69	4.85/5.49
15. Ung. M	180	6.08/4.80	5.51/4.89	5.87/4.80	5.59/4.80	5.80/4.53
16. Untreated control	180	6.85/5.95	6.88/6.12	6.87/6.22	6.32/5.61	6.24/6.14

MAL, methyl aminolevulinate; AFXL, ablative fractional laser, here fractional CO_2 laser; Ung. M, Unguentum M.
Presented are median accumulative fluorescence intensities (a.u.) with 25%- and 75% percentiles in dermis (D) and hair follicle (HF) epithelium for the 16 interventions. $n = 10$ frozen biopsies from each intervention were analyzed at five different skin depths from 120 to 1,800 μm (100 biopsies × 5 depths = 500 measures). Percentiles are illustrated on Figures 3 and 4.The fluorescence intensities of control interventions were comparable at the five skin depths in both HF and dermis ($P > 0.05$).

Finetek, Torrance, CA) and snap frozen. From each biopsy, horizontal sections of 12 μm thickness were collected at five different skin depths 120 μm (±12), 500 μm (±48), 1,000 μm (±48), 1,500 μm (±48), and 1,800 μm (±48).

Digital fluorescence microscopy was performed using a Nikon Eclipse TE 2000-S (Tokyo, Japan) with 415 nm excitation and 635 nm emission band-pass interference filters. The power of the excitation light was monitored with an external power meter (Nova II Ophir, PD300 sensor P/N 7Z02410, serial no. 542102, Laser Measurement Group, Tel Aviv, Israel) and kept stable at an intensity of 4.7 mW/cm^2 (±10%) and by fluorescence measurements on a standard plastic plate (Plexiglass F322, Rhom and Haas, Philadelphia, PA) 25,331 pixels (±10%). Bright field and fluorescence images were captured using a CCD camera (RT3™ slider, Diagnostic Instruments Inc., Sterling Heights, MI) with associated software (Spot Advanced™ Software). The digital images acquired were processed and analyzed as previously described by using Photoshop (version CS5.1, Adope Systems Inc., San Jose, CA) and MatLab (version 7.0.1, MathWorks, Natrick, MA) [9,10]. Regions of interest were selected on bright field microscopy images and then transferred to the corresponding fluorescence images. Mean dermal fluorescence intensities were calculated from 650 × 650 pixel (1,300 μm × 1,300 μm) squares centered around the single laser holes or at the center of images for non-laser interventions. Fluorescence from sebaceous glands, sweat glands, and HF was excluded from these calculations. The mean fluorescence intensities of HF epithelium were calculated from 50 × 50 pixel (100 μm × 100 μm) squares.

Statistics

The D'Agostino–Pearson normality test indicated significant deviations from normal distribution in some interventions, non-parametric analyses were therefore used. Descriptive data were presented as medians with 25% and 75% percentiles. The Mann–Whitney *t*-test was used for two-group comparisons and Kruskal–Wallis test for more than two-group comparisons. *P* values <0.05 were considered significant. Statistics was performed using PRISM® GraphPad, version 4.03 (GraphPad Software Inc., La Jolla, CA).

RESULTS

Dimensions of MTZs

The dimensions of laser channels were evaluated under light microscopy. Vertical sections detected ablation depths down to (i) 300 μm (37 mJ/MTZ) (ii) 1,400 μm (190 mJ/MTZ), and (iii) 2,100 μm (380 mJ/MTZ) corresponding to laser channels reaching (i) superficial dermis, (ii) mid dermis, and (iii) deep dermis/upper subcutaneous fat layer. Ablation widths in superficial dermis were for all three laser settings approximately 170 μm with surrounding coagulation zones of approximately 80 μm. Histological images of three representative MTZs are illustrated in Figure 1.

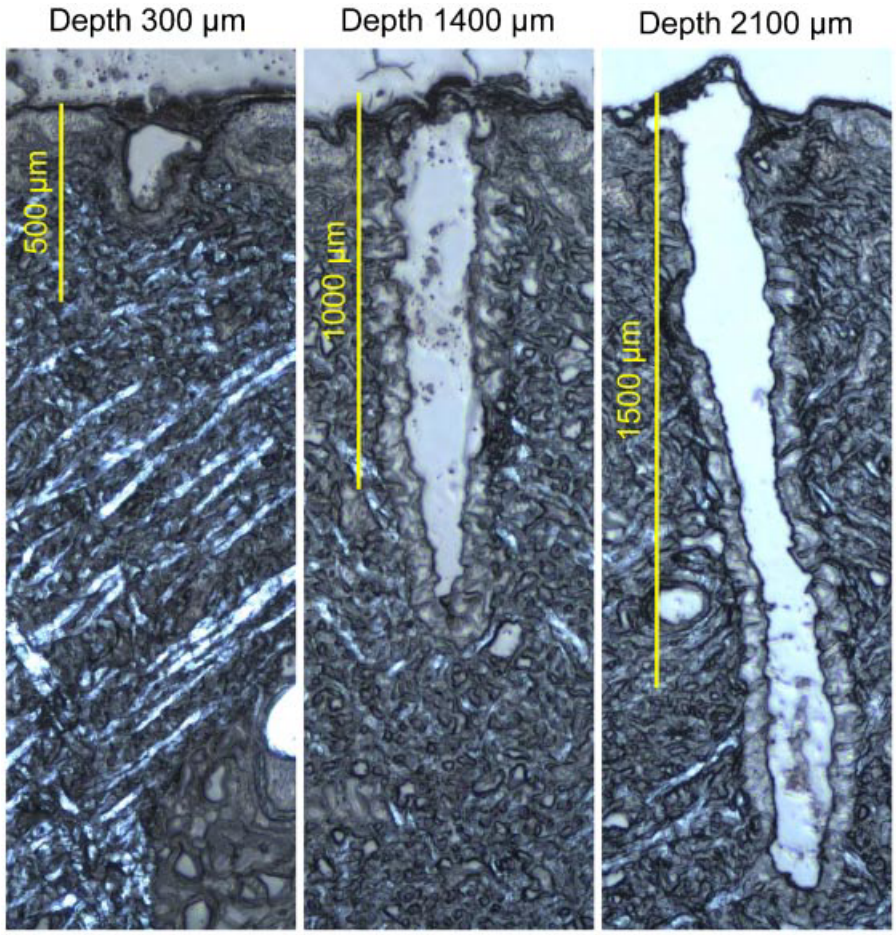

Fig. 1. Vertical sections of frozen biopsies. Images illustrate microthermal zones (MTZ) created with fractional CO_2 laser at three different settings, delivering total energy levels of 37, 180, and 380 mJ/MTZ. MTZs were ablated down to 300, 1,400, and 2,100 µm. Ablation widths in the superficial dermis were approximately 170 µm for all three laser settings with surrounding coagulation zones of approximately 80 µm.

Impact of Laser Channel Depth

After 180 minutes MAL incubation, fluorescence intensities were evaluated for three different intradermal laser channel depths (Table 1).

Surface fluorescence. In non-laser treated skin, surface fluorescence presented with speckled PpIX accumulation (Fig. 2). In laser treated skin, fluorescence accumulated around the individual MTZs with intensified speckled fluorescence of surrounding skin areas (Fig. 2). Surface fluorescence intensities reached similar levels independent of depth of laser channels (186, 192, and 177 a.u., respectively; $P = 0.19$, Table 2) and significantly higher fluorescence values were measured in laser treated than non-laser treated skin (186, 192, and 177 vs. 23 a.u.; $P < 0.0001$). Fluorescence intensities in untreated control, laser control and placebo cream test sites were significantly lower than corresponding MAL-exposed test sites ($P < 0.0001$). Fluorescence standards on photographs remained stable between interventions (Red-Tags, $P = 0.19$).

Fluorescence intensities throughout skin layers. Overall, laser treatment before MAL application significantly increased dermal and HF fluorescence intensities throughout all skin layers (120–1,800 µm; $P < 0.0001$; Table 1 and Fig. 3).

In HFs, similar fluorescence levels were measured at specific horizontal skin layers (120, 500, 1,000, 1,500, and 1,800 µm) independent of laser channels penetration depth ($P > 0.25$; Fig. 3A). Fluorescence intensities were stable in the vertical axis from superficial to deep skin layers for laser channels drilled into mid (1,400 µm) and deep (2,100 µm) dermis ($P = 0.67$ and 0.95, respectively), but fluctuated between skin layers for superficial laser

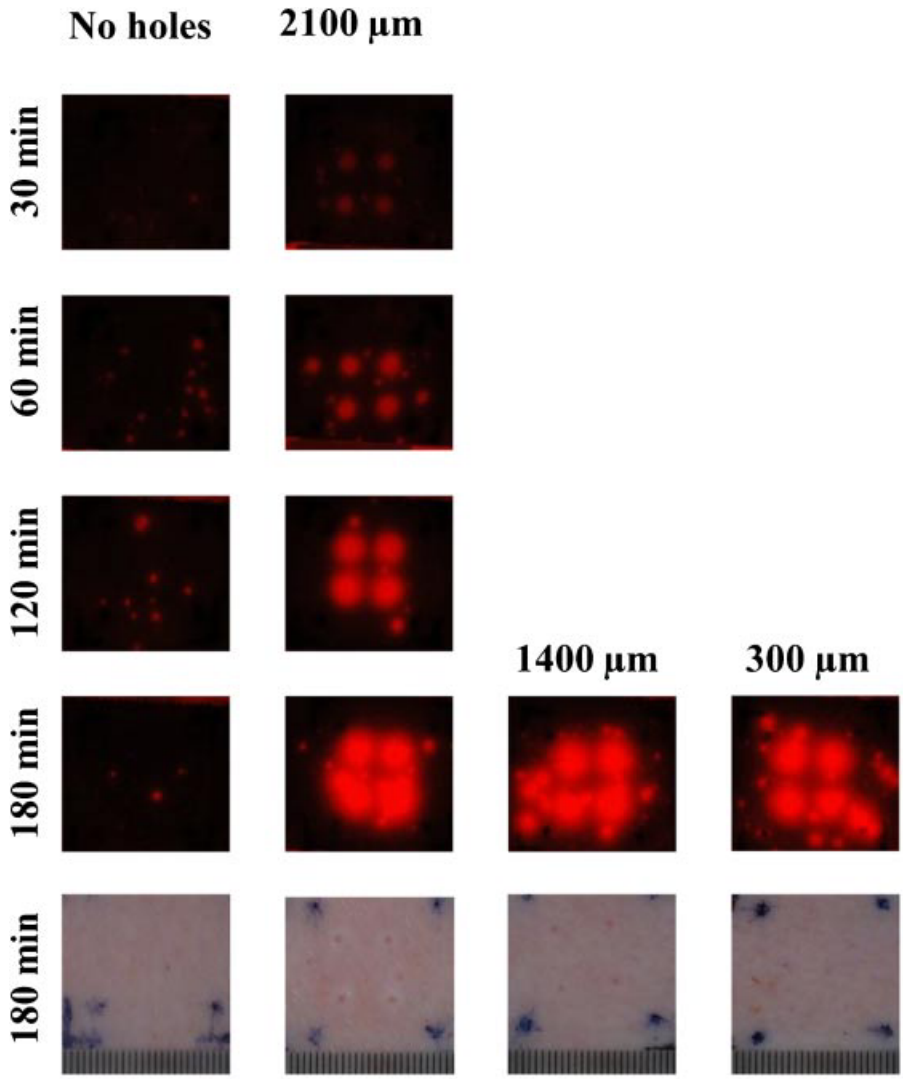

Fig. 2. Surface fluorescence images and corresponding clinical images of test sites pretreated with ablative fractional laser (AFXL) before application of methyl aminolevulinate (MAL). Surface fluorescence intensities were significantly increased by AFXL pretreatment and similar fluorescence levels were observed after 180 minutes MAL incubation, independent of laser channel depth. Further, the kinetics of PpIX accumulation was significantly accelerated in AFXL treated test sites. Clinical images after 180 minutes MAL incubation are illustrated and demonstrate similar skin reactions from laser treatments with three different laser settings used.

channels (300 μm; 120 and 1,800 μm depth <500, 1,000, and 1,500 μm depth; $P < 0.03$; Table 1 and Fig. 3A).

In dermis, fluorescence intensities were similar throughout specific horizontal skin layers independent of laser channels penetration depth ($P > 0.19$; Fig. 3B). In the vertical axis from superficial to deep skin layers fluorescence intensities decreased slightly independent of MTZ depth, but significance was not reached ($P > 0.05$; Table 1 and Fig. 3B).

Kinetics of Protoporphyrin IX Fluorescence

Fluorescence intensities were examined for 30, 60, 120, and 180 minutes incubation times in skin exposed to deep dermal AFXL channels (Table 1).

Surface fluorescence. PpIX fluorescence increased over time for all MAL interventions (30–180 minutes; $P < 0.014$) and saturation of the skin was therefore not observed within the time frame of 180 minutes incubation (Table 2). For all incubation times, surface fluorescence was significantly higher in laser treated than non-laser treated skin (30–180 minutes; $P < 0.0003$; Table 2 and Fig. 2). At 60 minutes after MAL application, laser-induced fluorescence intensities were significantly higher than corresponding non-laser treated skin at 180 minutes ($P = 0.0003$).

Fluorescence intensities throughout skin layers. Laser exposure significantly accelerated PpIX accumulation (Table 1). After 60 minutes MAL incubation, HF

TABLE 2. Median Surface Fluorescence Intensities

Intervention	Time (minutes)	Surface fluorescence (a.u.)
1. MAL + AFXL (37 mJ)	180	185.58 (179.69–195.84)
2. MAL + AFXL (190 mJ)	180	192.44 (180.08–204.66)
3. MAL + AFXL (380 mJ)	30	31.08 (28.26–34.32)
4. MAL + AFXL (380 mJ)	60	69.16 (63.52–73.42)
5. MAL + AFXL (380 mJ)	120	115.32 (111.20–128.23)
6. MAL + AFXL (380 mJ)	180	176.96 (157.38–191.17)
7. MAL	30	15.65 (14.56–18.04)
8. MAL	60	22.31 (19.40–34.84)
9. MAL	120	37.42 (27.59–48.76)
10. MAL	180	23.49 (17.85–32.78)
11. AFXL (37 mJ)	180	9.77 (7.66–12.25)
12. AFXL (190 mJ)	180	8.09 (7.72–8.73)
13. AFXL (380 mJ)	180	7.25 (6.69–9.50)
14. Ung. M + AFXL (380 mJ)	180	10.50 (8.68–12.61)
15. Ung. M	180	11.82 (11.29–12.64)
16. Untreated control	180	6.16 (5.71–6.82)

MAL, methyl aminolevulinate; AFXL, ablative fractional laser, here fractional CO_2 laser; Ung. M, Unguentum M.
Median skin surface fluorescence intensities (a.u.) with 25% and 75% percentiles. $n = 12$–16 for each intervention (total = 200 measures).

fluorescence intensities throughout all skin layers reached significantly higher values in laser-exposed skin than in intact unexposed skin (AFXL-MAL 60 minutes vs. MAL 60 minutes; $P < 0.04$). After 120 minutes incubation, dermal fluorescence intensities were higher in laser-treated than unexposed skin (AFXL-MAL 120 minutes vs. MAL 120 minutes; $P < 0.0002$; Table 1).

Down to a skin depth of 500 μm, laser exposure and 60 minutes MAL incubation induced significantly higher fluorescence intensities of HF than non-laser treated skin, incubated with MAL for 180 minutes (AFXL-MAL 60 minutes vs. MAL 180 minutes; $P < 0.02$; Fig. 4A). Throughout all skin layers, AFXL and 120 minutes MAL incubation induced significantly higher fluorescence intensities than non-laser exposed skin and 180 minutes incubation (HF and dermis; AFXL-MAL 120 minutes vs. MAL 180 minutes; $P < 0.01$; Fig. 4A and B).

Similar fluorescence levels were measured throughout skin layers for all incubation times (120–1,800 μm depth at 30, 60, 120, and 180 minutes; $P > 0.05$; Fig. 4A and B).

DISCUSSION

This *in vivo* study for the first time substantiates that AFXL-assisted accumulation of PpIX is independent of the depth of intradermal laser channels. Accentuated kinetics of AFXL-assisted PpIX accumulation was quantified from skin surface to deep dermal skin layers, indicating that photosensitizer incubation time may be reduced from AFXL pretreatment. These results raise perspectives for future optimized treatment parameters for AFXL-assisted MAL PDT.

The main barrier for uptake of topical photosensitizers is the outermost skin layer, stratum corneum [28]. Further potential epidermal barriers comprise keratinocyte to keratinocyte junctions and the basal lamina, whereas barriers for distribution within dermis are considered of less importance. However, the importance of intradermal laser channel depth for AFXL-facilitated drug delivery has so far not been investigated. This study presents the first data that varying penetration depth of laser channels from superficial to deep dermis does not affect MAL-induced accumulation of PpIX in HF and dermal compartments. Similar levels of PpIX accumulation were documented throughout all skin layers after drilling laser channels from 300 to 2,100 μm depth. We therefore conclude that dermis does not seem to impose a limitation for distribution of a rather small (182 Da) lipophilic molecule (MAL).

To our knowledge, no former studies have evaluated the importance of depth of fractional CO_2 laser channels for delivery of topical photosensitizers. In literature, two previous studies have investigated varying depths of Er:YAG laser channels for delivery of the topical photosensitizer 5-aminolevulinic acid, ALA [11,12]. These two studies drilled MTZs with a maximum penetration depth of approximately 70 μm, presenting far more superficial laser channels than applied in our study (300–2,100 μm). Lee et al. [12] used laser settings of 2 and 3 J/cm^2 to generate MTZs that disrupted the cornified layer of the skin but did not penetrate through stratum corneum. Data demonstrated that an increase in laser fluence led to an enhanced permeation of ALA. Forster et al. [11] presented data using laser fluencies from 4 to 24 J/cm^2. Energy levels from 4 to 8 J/cm^2 drilled laser channels within stratum corneum and 12–24 J/cm^2 drilled channels of varying intraepidermal penetration depths. Forster et al. found that energy levels above 6 J/cm^2 did not facilitate a

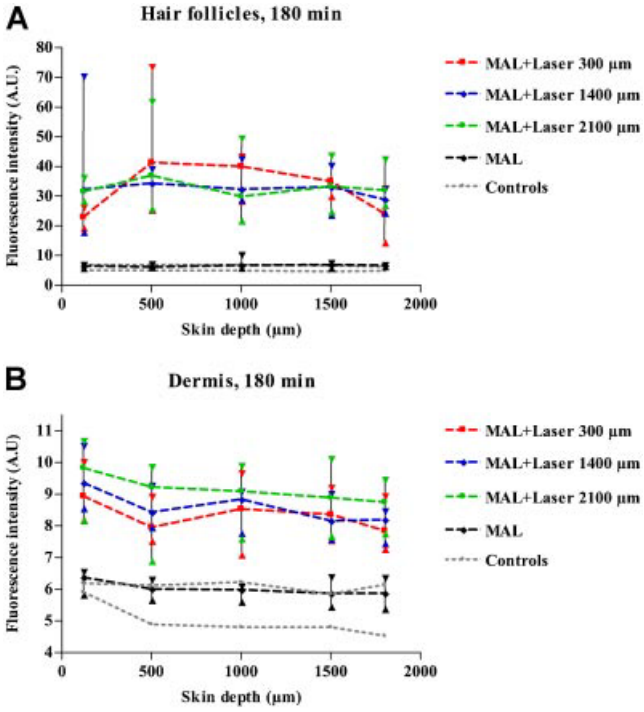

Fig. 3. Fluorescence intensities at five different skin depths are illustrated for (**A**) hair follicles and (**B**) dermis after180 - minutes MAL incubation. Data are presented as medians with 25% and 75% percentiles. Presented are fluorescence levels in non-AFXL treated skin and skin pretreated with AFXL at three different intensities, creating MTZs with ablation depths down to 300, 1,400, and 2,100 µm. Fluoresce images were analyzed at five different skin depths of 120, 500, 1,000, 1,500, and 1,800 µm. Each intervention includes data from $n = 10$ frozen biopsies. Note the different scales for fluorescence in A and B. Medians of control interventions (intervention #5−10, Table 1) are presented as the area between the two gray lines.

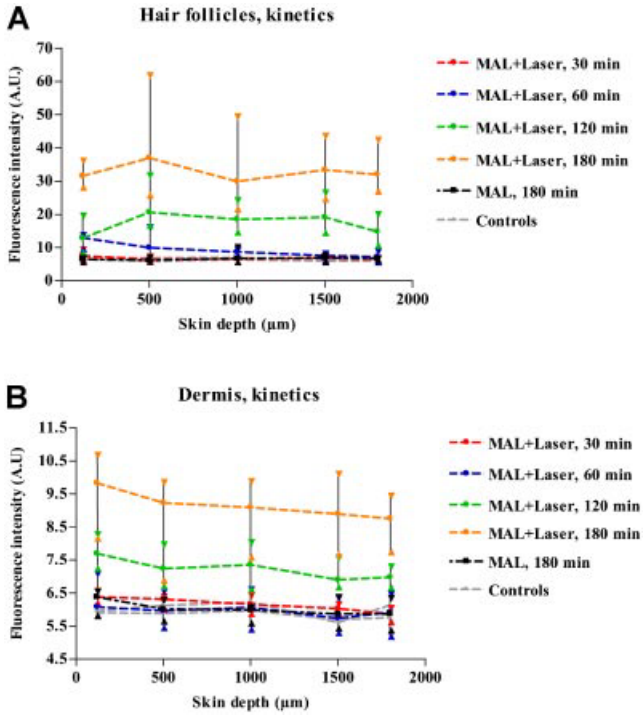

Fig. 4. Fluorescence intensity levels are illustrated for (**A**) hair follicles and (**B**) dermis, after 30, 60, 120, and 180 minutes MAL incubation. Data are presented as medians with 25% and 75% percentiles. AFXL treatment at an energy level of 380 mJ/MTZ created MTZs with ablation depths of approximately 2,100 µm. Fluoresce images were analyzed at five different skin depths of 120, 500, 1,000, 1,500, and 1,800 µm. Each intervention includes data from $n = 10$ frozen biopsies. Note the different scales for fluorescence in figure A and B. Medians of control interventions (intervention #13 + 16, Table 1) are presented as two gray lines.

further increase in ALA delivery. Taking the present data from our study into account, studies conducted on animal models indicate that once stratum corneum is disrupted by AFXL treatment, there will be no further benefit from drilling deeper laser channels for the delivery of topical photosensitizers. This information is important from a clinical perspective, since the combination of minimal skin damage and high efficiency is attractive in PDT. Future clinical studies are needed to substantiate on this assumption and studies comparing superficial epidermal with superficial dermal laser channels is required.

The approved MAL incubation time is 3 hours for the treatment of actinic keratoses with PDT and red light [3–5]. This study substantiates that AFXL-assisted delivery of MAL significantly accelerates PpIX accumulation in the skin. In laser-treated skin, surface PpIX fluorescence was already after 60 minutes MAL incubation higher than fluorescence levels in non-laser treated skin after 180 minutes incubation. Throughout the entire skin, from superficial to deep skin layers (120–1,800 µm), fluorescence intensities after 120 minutes MAL incubation were significantly higher than non-laser exposed sites after 180 minutes incubation (Fig. 4). The clinical perspective of these data is to shorten MAL incubation time, which is obviously advantageous for both patients and physicians. The optimal incubation time may need to be adjusted to the specific type of lesion being treated. Thus, for very superficial lesions, an incubation time of 60 minutes may be sufficient while for thicker lesions incubation times may have to be extended to 120 minutes.

By disrupting the skin barrier AFXL reduces the time for uptake and distribution of MAL. The MAL-induced PpIX fluorescence showed no significant decrease with depth in the skin at any incubation times. At first this seems counter-intuitive, since there must be a decreasing concentration gradient of MAL from the skin surface to deep dermis. The best explanation for these findings is

that that after AFXL exposure, uptake, and distribution of MAL are not rate-limiting steps for PpIX accumulation, even in deep dermis. Therefore, other rate limiting steps are important to recognize. The metabolic conversion of MAL to PpIX is slow. After distribution of MAL it must actively be taken into cells, and then into mitochondria where production of PpIX is part of the complex pathway for heme synthesis [8,29,30]. When intracellular iron stores are exhausted, PpIX finally begin to accumulate [30]. In our study, the constant porphyrin fluorescence with skin depth, and independence from laser channel depth, are consistent with rapid delivery of enough MAL to saturate the slow PpIX synthesis pathway, throughout the entire skin. Under this condition, a concentration gradient of MAL inside the skin has no effect on PpIX accumulation, as we observed. The time frame for PpIX synthesis must therefore be taken into consideration for minimum MAL incubation times.

It is an important limitation to this *in vivo* model that results are based on normal pig skin instead of dysplastic or malignant human skin lesions. Uptake and distribution of MAL could potentially vary between normal skin and tumors. Moreover, the selectivity of PDT for dysplastic and neoplastic tissue could be affected by the generalized increased PpIX accumulation induced by AFXL. In former studies from this group, intensified PpIX fluorescence levels have been observed in areas of premalignant and NMSC lesions compared to adjacent normal tissue after AFXL pretreatment [26,27]. However, to clarify differences between PpIX distribution in normal skin and diseased skin future studies are needed. For the purpose of clinical relevance, we performed this study using MAL in the formulation and concentration commonly used for PDT. We found that AFXL-assisted delivery of the clinically used concentration of MAL drives the rate of PpIX synthesis into saturation, throughout the entire porcine skin. When treating human NMSC with MAL-PDT, it is highly desirable to ensure deep penetration of MAL. Our study strongly suggests that AFXL pretreatment will lead to much greater, more uniform, and anatomically deeper expression of porphyrins, which are the ultimate photosensitizer during PDT with MAL. We intend to examine this hypothesis in clinical studies.

This *in vivo* study substantiates that different intradermal penetration depths of fractional CO_2 laser channels are equally efficient to accumulate PpIX throughout skin layers. Furthermore, pretreatment with AFXL accelerates the kinetics of PpIX fluorescence, which raises clinical perspectives for shortened MAL incubation times.

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